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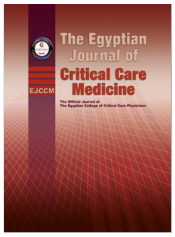

ORIGINAL ARTICLE

Role of Phosphorylated Neurofilament H as a diagnostic and prognostic marker in traumatic brain injury

Moh Omar Ghonemi [a,*], Amany Ahmed Rabah [b], Hamdy Mohamed Saber [c], Wahid Radwan [a]

[a] *Department of Critical Care, Cairo University, Egypt*
[b] *Department of Neurology, Cairo University, Egypt*
[c] *Department of Critical Care, Beniswif University, Egypt*

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KEYWORDS

Neurofilament H;
Traumatic brain injury;
Rankin score;
Glasgow coma score;
Acute brain insults

Abstract *Background and purpose:* One of the main drawbacks in the management of patients with traumatic brain injuries (TBI) is the absence of a widely available and rapid diagnostic test. The objective of our study was to assess whether Phosphorylated Neurofilament H (pNF-H) might provide useful diagnostic information as an indication of axonal injury in the early evaluation of such patients and whether levels of the pNF-H correlated with different clinical variables.

Methods: A total of 30 patients presenting to the critical care department of Cairo University diagnosed with traumatic brain injury were prospectively studied. Blood samples for pNF-H were assayed on admission and after 7 days. Neurofilament levels correlated with Glasgow coma scale and CT findings on admission and after 7 days. Rankin score at 3 months was used to detect the degree of disability.

Results: Phosphorylated Neurofilament H levels showed a negative correlation with GCS on admission and after 7 days in traumatic brain injury; hence higher neuromarker levels were associated with lower GCS on admission and after 7 days. There was a negative correlation between neurofilament levels and Marshal CT scores on admission and after 7 days ($r = 0.56.0.4$) hence higher neurofilament levels correlated with worse CT findings. Patients who died or had the greatest Rankin 6 and 5 after 3 months had the highest levels of pNF-H on admission and after 7 days. The cut off level of pNF-H to detect death and disability was 35 pg/ml on admission (sensitivity 82%,

* Corresponding author. Tel.: +20 1112093839.
E-mail address: elghonemi@yahoo.com (M.O. Ghonemi).

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specificity 78%) and was 11 pg/ml after 7 days (sensitivity87%, and specificity 92%).

Conclusion: Phosphorylated Neurofilament H can be used as a diagnostic and prognostic marker in patients with TBI as seen by the presence of significant correlations between the marker levels and different clinical and radiological tools.

Introduction

Following CNS injury, certain proteins are released from neurons. Many studies have identified such proteins like S-100 protein, Glial Fibrillary Acidic Protein (GFAP) and Neuron specific enolase (NSE). However none of those markers have shown strong correlations with clinical and radiological findings in patients with TBI. There has been a growing appreciation that many kinds of CNS injury and disease states are the result of axonal injury and degeneration [1,2]. Accordingly, a convenient method of detecting ongoing axonal loss might be particularly useful experimentally and clinically. The perfect marker to detect axonal injury should have several properties; it should be specific to axons, it should be profuse enough so that it can be readily detectable after the significant dilution that occurs following release into blood, and it should be resistant to proteases so that it is not broken down prior to or following release.

Over the last years, many strides have been taken in the characterization of Neurofilament proteins and their functions. Early studies by Hsieh et al. have shown that phosphorylation of neurofilaments is required for the proper functions of axons by regulating axon diameter [21]. The Neurofilament H (pNF-H) protein sequence contains unusual tandemly repeated 6–8-amino-acid sequences centered on the sequence lysine-serine-proline. The serine residues are phosphorylated [3] and are axon specific [4]. This phosphorylated form of NF-H (here referred to as pNF-H) is known to be more resistant to calpain and other proteases [5,6] Also, pNF-H is highly immunogenic, and the multiple repeated phosphorylated sites are an excellent target for antibody-based assays. Taken together, these facts suggest that pNF-H might be a good candidate for a biomarker of axonal injury.

Patients and methods

Study design

This is a randomized study that was prospectively conducted on 30 patients with TBI presenting to the Critical Care Department Cairo university Hospitals in the period from January 2010 to January 2011. Informed written consents had been obtained from the relatives and the study was approved by the Hospital's Ethics Committee.

Inclusion criteria

Thirty patients with isolated traumatic brain injury defined according to the Head Injury Interdisciplinary Special Interest Group of the American Congress of Rehabilitation Medicine [16] by having at least one of the following: any period of loss of consciousness, any loss of memory for events immediately before or after the trauma, any alteration in mental state at the time of the accident focal neurologic deficits which may or may not be transient, and abnormal CT scan findings.

Exclusion criteria

The following patients were excluded:

- Patients with chronic neurological disease
- Patients with seizure activity
- Patients with renal impairment
- Patients with Multi-organ Failure Syndrome
- Patients with Multiple trauma
- Patients younger than 18 years and older than 65 years
- Patients who were receiving or those candidates for thrombolytic therapy.

Methods

- Full history taking from the patients, relatives or witnesses with stress on the onset of neurological symptoms.
- Complete general and focused neurological examination.
- Blood samples were taken for routine labs and for pNF-H levels. Samples were taken within the first 24 h (pNF-H1) and after 7 days (pNF-H2).
- For each patient a non-contrast CT brain (NCCT) was done on admission and after 7 days. Image review was independently performed on a workstation radiologists or neurologists. Marshall CT score was used to assess CT findings on admission (MARSHALL1) and after 7 days (MARSHALL2).
- Glasgow coma scale was calculated on admission (GCS1) and repeated after 7 days (GCS2).

Outcome analysis: Rankin score

Rankin score was used to assess patients' outcome after an interval of 3 months. Each patient was given a number from 1 to 6 according to the level of disability as shown in Table 1 [19].

Neurofilament H assay method

Blood samples were drawn from each patient on admission and after 7 days. The BioVendor Human Phosphorylated Neurofilament H ELISA, standards, quality controls and samples were placed and left in microplate wells that contained chicken polyclonal anti-pNF-H antibody. One hour later, detection rabbit polyclonal anti-pNF-H antibody was added and incu-

Table 1 Rankin Score.

6 = Death	
5 = Severe disability.	Bedridden, incontinent, and requiring constant nursing care and attention
4 = Moderately severe disability	Unable to walk without assistance, and unable to attend to own bodily needs without assistance
3 = Moderate disability;	Requiring some help, but able to walk without assistance
2 = Slight disability	Unable to carry out all previous activities but able to look after own affairs without assistance
1 = No significant disability	Able to carry out all usual duties and activities

bated with captured pNF-H for 60 min. After another washing, horseradish peroxidase conjugated antibody against rabbit antibody was added. After 60 min incubation and the last washing step, the remaining conjugate was allowed to react with the substrate solution. The reaction was stopped by addition of acidic solution and absorbance of the resulting yellow product was measured.

Data analysis

Pearson correlation analysis of data was performed using Statistical Package for the Social Sciences (SPSS) software. The association of subject characteristics to pNF-H levels was studied with multiple regressions. The pNF-H data were square root transformed to effect normality of distribution of residuals. Relationships between the square roots of pNF-H were investigated within groups with Pearson correlation and the two-sample *t*-test. Analysis of correlation was used to assess the different relationships between pNF-H and other variables.

Results

The study of demographic characteristics of the patients groups showed that the mean age of patients in the groups studied was 36.5 with a range of 19–55 years. There was a total of 17 male patients and 13 females.

Mean Neurofilament H levels in the study group

The mean Neurofilament level on admission (pNF-H1) was 47 ± 25.8 pg/ml in Group A and the mean Neurofilament H level after 7 days (pNF-H2) was 148 ± 90.3 pg/ml i.e. the Neurofilament level was significantly higher after 7 days ($p < 0.005$).

Analysis of outcome: Rankin score

There was an apparent increase in mean Neurofilament levels in patients with the greatest disability (Rankin 5) and those who died (Rankin 6). Patients with the least disability (Rankin 1 and 2) showed the lowest mean Neurofilament levels on admission and after 7 days. Patients with higher levels of Neurofilament H on admission and after 7 days showed a greater Rankin score after 3 months and hence a greater disability ($p < 0.005$). Hence Neurofilament H levels were significantly higher in patients with poor outcome (Fig. 1).

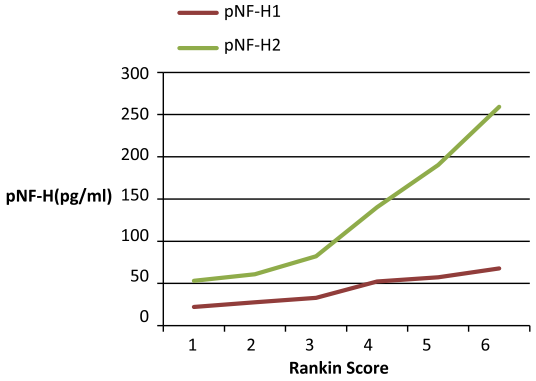

Figure 1 Relationship between Rankin Score after 3 months and Neurofilament levels in the study population.

Receiver-operating characteristic (ROC) curves showing discriminative abilities of Neurofilament H for predicting outcome after 3 months

On admission

On admission, the ROC curve showed cut-off point of 35 pg/ml for pN-FH level to predict severe disability or death with sensitivity 82.1% and specificity 78.4%, positive predictive value 74.4% and negative predictive value 14.9% with Area Under Curve (AUC 87.1%). Hence patients with Neurofilament H levels of 35 pg/ml or more on admission had a worse Rankin score after 3 months (Fig. 2).

After 7 days

One week later, the ROC curve showed cut-off point of 111 pg/ml for pNF-H to predict severe disability or death with sensitivity 87.2% and specificity 92.2%, positive predictive value 89.5% and negative predictive value 9.6% with Area Under Curve (AUC 95.7%). Hence patients with Neurofilament H levels of more than 111 pg/ml after 7 days had a worse Rankin score after 7 days (Fig. 3).

Correlation between GCS and Neurofilament H levels

As shown by the scatter diagram there was a strong negative correlation between the level of Neurofilament H and the Glasgow Coma Scale in patients with traumatic brain injury on admission ($r = 0.66$ with a $p < 0.005$). This applies also after 7 days ($r = 0.78$, $p < 0.005$). In other words, the higher pNF-H levels correlated with lower GCS (Fig. 4).

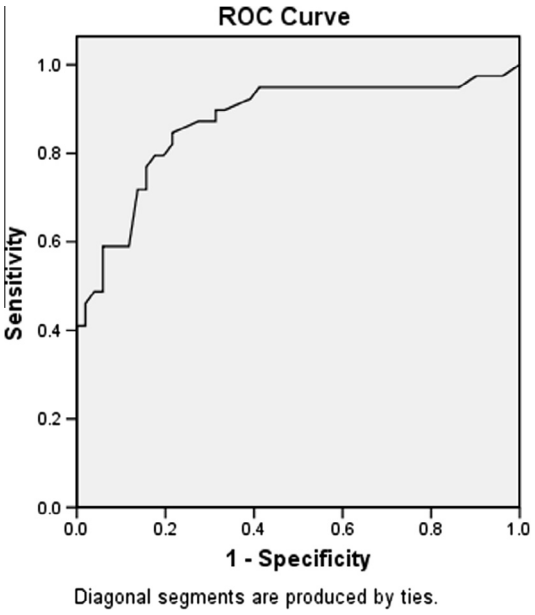

Figure 2 ROC curve on admission.

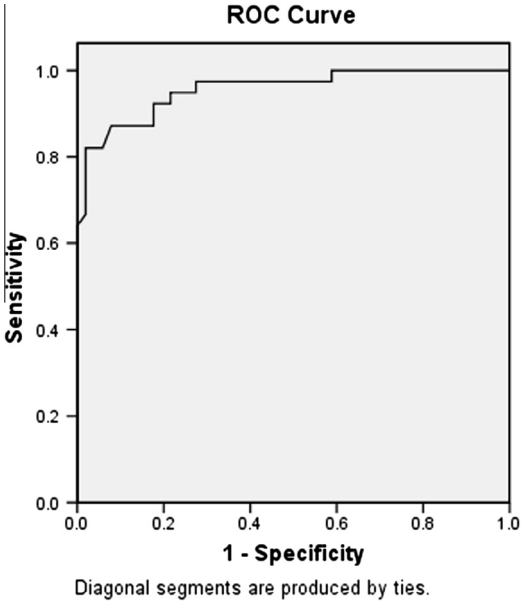

Figure 3 ROC curve after one week.

Mean MARSHAL CT score in the study group

The mean marshal score in group A was 2.1 on admission (Marshal1), and was 3.7 after 7 days (Marshal2) (Fig. 5).

6- Correlation between Neurofilament H and Marshall score

There was a significant positive correlation of 0.56 between the mean Marshal score and the mean Neurofilament H level both on admission and after 7 days ($r = -.56.0.4$ $p < 0.005$) In other words, the higher the level of Neurofilament the higher the Marshal score (Fig. 6).

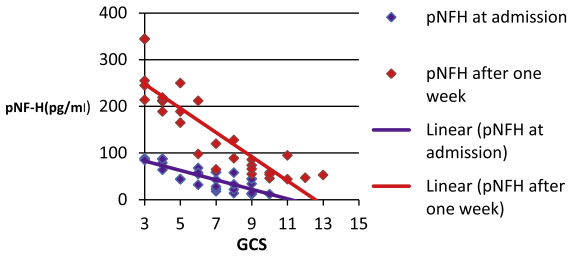

Figure 4 Correlation between pNFH and GCS.

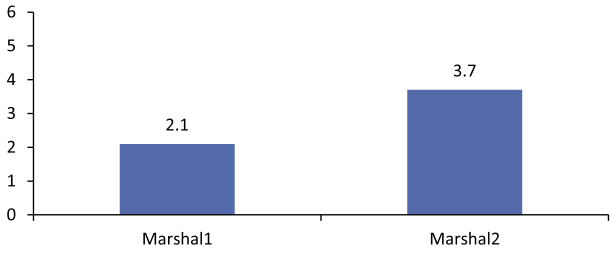

Figure 5 Mean MARSHALL scores.

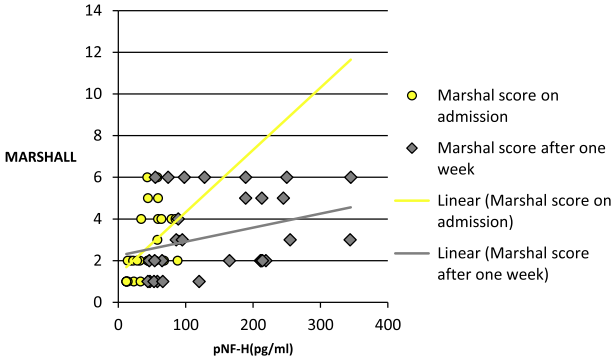

Figure 6 Correlation between pNF-H and MARSHALL.

Discussion

One of the main problems in patients with TBI is the absence of a blood marker that can be collected easily to aid in diagnosis and follow up of these patients. Numerous scientists have studied various proteins for this purpose but results were not promising.

The best example for a diagnostic and prognostic marker is the cardiac troponin test. Development of techniques to detect the cardiac isoenzymes of troponin with high sensitivity led to the ultimate revision of the clinical definition of myocardial infarction to elevate the role of biomarkers in diagnosis. The main property of the troponin test that led to its widespread use was a nearly absolute specificity for myocardial tissue (unlike creatine kinase), as well as high sensitivity. A biomarker with similar properties to detect acute brain insults would be of great use [22].

Phosphorylated Neurofilament H (pNF-H) is a large protein with a molecular weight of 190–210 kD depending on the degree of phosphorylation. Following neuro-axonal injury

pNF-H is released into the extracellular fluid from where it can be measured using ELISA. From the extracellular fluid it diffuses into the CSF compartment. From the CSF, pNF-H reaches the blood via the blood CSF barrier at the lumbar level or it may diffuse directly through the cortical arachnoid villi to the blood stream [20].

In this study, we have demonstrated that pNF-H can be detected in the blood of patients with traumatic brain injuries. The presence of Neurofilaments in blood indicated damage of neurons after such injuries. The importance of pNF-H lies in the fact that it could be detected in the blood of TBI patients, which makes such marker a convenient and simple indicator of brain damage, in contrast to other markers measured in the CSF. Blood collection is more practical and safer than CSF sampling suggesting that analysis of blood for pNF-H could be a useful clinical tool to conveniently assess axonal damage.

Recent data have emphasized that the morphology of vertebrate neurons renders the axons sensitive to mechanical and metabolic damage and a major portion of the pathophysiology following brain injury is due to axonal damage [1]. Therefore, a convenient blood assay of axonal loss could be of great utility.

Our data showed negative correlation between the GCS and pNF-H levels both on admission and after 7 days. pNF-H levels also correlated with CT findings. In traumatic brain injury patients, levels showed an inverse relationship with MARSHAL CT scores.

Rankin score was used to assess outcome after 3 months. Patients with higher levels of Neurofilament H on admission and after 7 days showed a greater Rankin score after 3 months and hence a greater disability ($p < 0.005$). There was an apparent increase in mean Neurofilament levels in patients with the greatest disability (Rankin 5,) and those who died (Rankin 6,). Patients with the least disability (Rankin 1 and 2) showed the lowest mean Neurofilament levels on admission and after 7 days. Hence Neurofilament H levels were significantly higher in patients with poor outcome. ROC curve showed cut-off point for NFH level at admission to predict severe disability or death was 35 pg/ml and 111 pg/ml after 7 days.

Several other proteins have been proposed as potential biomarkers in TBI [9] For example, the small Ca^{2+}-binding modulator protein S-100β has been extensively studied in both animal models of TBI and human TBI victims [9]. While S-100β has been shown to be elevated following TBI in humans, its specificity to brain injury was debated since it appears to be elevated in humans with other forms of trauma not related to the brain. In addition, S-100β levels have been shown to be elevated following femoral fractures without any accompanying brain injury [10] and are elevated in long distance runners [11]. Although pNF-H was elevated post traumatic brain injury in our study and correlated with the level of consciousness and CT findings, we still do not have any data about other causes that will increase neurofilaments in blood and whether our marker – pNF-H – is more specific to axonal damage than other markers.

However, patients included in our study suffered only trauma to the head, and patients with multiple trauma or those with multiorgan dysfunction were excluded. Thus further studies may be needed to assess the degree of specificity of pNF-H.

Previous studies have also examined serum levels of Cleaved tau protein (c-tau) as a marker of TBI in both humans and animal models. However, the level of c-tau in the serum of injured humans and animals is significantly less than pNF-H. Furthermore, tau is not neuronal specific; it is expressed in nonneuronal tissues such as the heart, skeletal muscle, lung, kidney, and testis [7,12]. Again further studies will be required before we understand if similar problems will occur with the use of pNFH protein as a biomarker and if it will be elevated in other non neurological conditions.

Interestingly, Anderson J studied Neurofilaments in rats subjected to different forms of cortical contusion and were stratified according to the degree of trauma into moderate and severe cortical contusions. The level of serum Neurofilaments detected correlated with the severity of the brain injury. The peak of Neurofilaments was seen at 2 days post-injury in all groups, with the severe injury group showing the highest amounts of pNF-H. He stated that "*It will be of interest to compare the results obtained here on rats with the results of cortical injury on larger mammals and humans. Larger mammals have much thicker and usually folded cortices containing a greater proportion of white matter that is expected to be rich in Neurofilaments containing axons. In fact, about 42% of the human neocortex is white matter compared to less than 10% in the rat and other small lissencephalic mammal*" [23]. Our study results can be used to complement such results showing the Neurofilaments can be detected in humans suffering from different forms of TBI and can be correlated with various clinical and radiological markers.

As mentioned above, if pNF-H is located solely in axons, axonal loss is a major problem in many kinds of human neurological damage and disease states, such as TBI, multiple sclerosis, and amyotrophic lateral sclerosis [1]. This can be problematic in patients with chronic CNS disease. However, in our study, we excluded patients with any neurological disorders other than traumatic brain injury thus we can say that increase in levels of pNF-H in our population was solely due to acute brain injury and not due to chronic CNS diseases.

Conclusion

Blood levels of pNF-H were quantifiable in patients with traumatic brain injury on admission to the ICU and after 7 days. The blood levels were significantly higher levels in patients with poor outcome as compared with those with good outcome in the first 3 months following TBI. Phosphorylated Neurofilaments levels showed significant correlations with the level of consciousness and CT findings in such patients. Further studies will be needed to complement our results in larger patients' samples and more importantly to elucidate other non cranial causes of increased pNF-H level in serum. A more detailed study of the pattern of rise and decline of pNF-H in patients with traumatic brain injury will be complementary to our study. Thus it is too early to recommend the routine use of plasma pNF-H in this context for clinical decision-making.

Authors' contributions

W.M.R., A.M.R., H.M. and M.O.E. made substantial contributions to conception and design of the study and were in-

volved in drafting the manuscript and made substantial contributions to the acquisition of data, its analysis, and interpretation. All authors read and approved the manuscript for publication.

Competing interests

The authors declare that they have no competing interests and there is no conflict of interests related to this study.

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